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# Glycosaminoglycans in *Anodonta californiensis*, a Freshwater Mussel

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**Abstract.** The synthesis of glycosaminoglycans (GAG) in a freshwater mussel was studied in organ culture using labeled precursors. The major GAGs synthesized were determined and characterized by chemical and enzymatic methods. They were shown to be heparin and an unusual type of heparan sulfate. Gills produced about 50% of each polymer; mantles synthesized little heparin and mostly the heparan-sulfate-like compound, which is similar to a GAG isolated previously from lobsters. No significant amounts of chondroitin sulfates were present.

Histological data showed that the sulfate-labeled GAGs were present mainly in exterior pericellular and basement membrane locations of gills and mantle. That is, they would be in contact with the external aqueous environment, suggesting a potential role in calcium transport and storage.

## Introduction

Glycosaminoglycans (GAG) constitute a group of highly charged complex polysaccharides that have a remarkable distribution in organisms. They are present from prokaryotes to vertebrates, and their main structural features appear to have been conserved during evolution. Their occurrence and distribution have been widely studied in animal species (Rahemtulla and Løvtrup, 1974; Cassaro and Dietrich, 1977; Nader *et al.*, 1983), and most of the six or so GAGs are represented in a variety of organisms. One unusual exception is heparin, which is present in some invertebrates (De Meio *et al.*, 1967; Rahemtulla and Lovtrup, 1975), and appears only sporadically in vertebrates but, with some exceptions (Gomes and Dietrich, 1982; Hovingh *et al.*, 1986), is widely distributed in mammals.

Various important biological functions have been ascribed to GAGs (Kjellen and Lindahl, 1991)—many well-documented, others speculative. Heparin is an excellent anticoagulant when used pharmacologically, but its biological role in higher organisms is uncertain. Though heparin interacts with such biologically active materials as growth factors, cell receptors, and matrix components, its absence from the *in vivo* locations where interaction is plausible has cast doubt on the biological significance of those interactions. The heparin isolated from species of marine clams and mussels (Pejler *et al.*, 1987) has structural features and anticoagulant activity identical to the mammalian polysaccharide. The presence and potential function of heparin in clams and mussels may provide a clue to its role in vertebrates because structural consistency may imply some functional consistency.

This study had two objectives: first, to determine the presence of heparin or other GAGs in a freshwater mussel; second, to examine the exact location of these polymers in the organism as an initial step toward identifying a potential role.

## Materials and Methods

*Anodonta californiensis* Lea was collected in western Utah in autumn and spring. The animals were either utilized immediately or kept in an aquarium for up to 6 months. Organ cultures were performed in Minimum Essential Eagle Medium (Modified) with Earle's salts and glutamine (Flow Laboratory, McClean, Virginia); the pH was adjusted to 7.2 with sodium bicarbonate. The medium contained gentimycin sulfate (80 μg/ml) and 10% bovine calf serum (Orvine Laboratory, Santa Ana, CA) and was sterile-filtered before use.

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Abbreviations: GAGs, glycosaminoglycans.

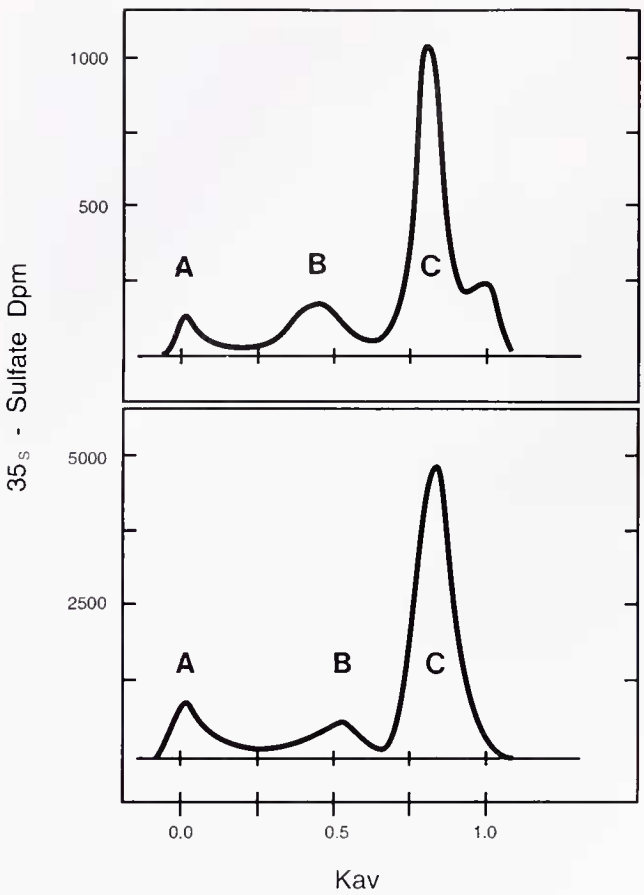



**Figure 1.** Sepharose CL-4B. Chromatography of labeled GAGs isolated from organs. Top: mantle. Bottom: gills. $K_{av}$ of heparan sulfate with molecular weight of 11,000 daltons is 0.58.

Radiolabeled sulfate ($Na_2[^{35}S]O_4$, 370 mCi/m*M*), [$^3$H] glucosamine (30 mCi/m*M*), and sodium borotritide were obtained from NEN (Burbank, CA). Pronase and chondroitinase ABC were obtained from Sigma (St. Louis, MO). Heparinase and heparitinase were prepared as described previously (Linker and Hovingh, 1972); they were free of other lyases, disaccharidase, and sulfatases. For the identification of isolated GAGs, the purified heparinase and heparitinase were used at 12 μg of enzyme protein per milliliter of 0.05 *M* phosphate buffer pH 7.0, at 25°C for the heparinase and at 37°C for the heparitinase. The solutions were incubated for 16 h. Chondroitinase ABC was used at 5–10 units/ml of the same buffer at 37°C and incubated for 16 h. Substrates were at 10 mg/ml. The low-pH nitrous acid method (Shively and Conrad, 1976) was also used for identification.

DEAE Sephacel, Sepharose Cl-4B, Sephadex G-50, G-25, and G-10 were obtained from Pharmacia (Piscataway, NJ) and Bio Rad AG 1-X2 from Bio Rad (Richmond, CA).

### *Chromatography and electrophoresis*

Columns of Sepharose Cl-4B (1100 × 10 mm) were eluted with 0.1% SDS in pH 7.5 Tris buffer containing protease inhibitors (10 m*M* EDTA, 10 m*M* N-ethyl-maleimide, and 5 m*M* benzamidine HCl). Sephadex G-50 and G-25 columns were eluted with 0.2 *M* NaCl in 10% ethanol and Sephadex G-10 columns with 10% ethanol in water. DEAE Sephacel columns were eluted stepwise in increments of 0.2 *M* NaCl starting with 0.2 *M* NaCl to 1.0 *M* NaCl in the presence of 1% Triton X-100 in 4 *M* urea with the protease inhibitors described above. Bio Rad AG1 × 2 columns (30 × 10 mm) were eluted with 0.5 *M*, 1.0 *M*, 1.25 *M*, and 2.0 *M* NaCl.

Electrophoresis was carried out on cellulose acetate strips in pyridine: formic acid (Hovingh and Linker, 1982) at pH 3.0 for 15 min at 50 mA. Enzymatic and nitrous acid breakdown products were identified by paper elec-

**Table I**

*Distribution of $^{35}$S-sulfate labeled polymers from gills and mantles after elution from sepharose Cl-4B columns: a summary of three experiments*

| | Peak A | | | Peak B | | | Peak C | | |
|---|---|---|---|---|---|---|---|---|---|
| | Percent of sulfate label | $K_{av}$* | $K_{av}$ after alkali | Percent of sulfate label | $K_{av}$ | $K_{av}$ after alkali | Percent of sulfate label | $K_{av}$ | $K_{av}$ after alkali |
| *Mantle* | | | | | | | | | |
| Expt. I | 9 | 0.0 | 0.0 | 19 | 0.47 | 0.4–0.80 | 72 | 0.80 | |
| Expt. II | 8 | 0.0 | 0.06–0.83 | 8 | 0.50 | 0.54–0.77 | 84 | 0.87 | 0.84 |
| Expt. III | 12 | 0.17 | 0.76 | 43 | 0.53 | 0.76 | 45 | 0.73 | 0.76 |
| *Gills* | | | | | | | | | |
| Expt. I | 12 | 0.0 | 0.0–0.8 | 9 | 0.52 | 0.80 | 79 | 0.85 | |
| Expt. II | 7 | 0.0 | 0.6–0.4 | 8 | 0.52 | 0.45–0.74 | 85 | 0.87 | 0.81 |
| Expt. III | 9 | 0.03 | ND | 32 | 0.53 | ND | 59 | 0.87 | 0.76 |

* A sample of an average commercial heparin had a $K_{av}$ of 0.6–0.93, which corresponds to a molecular weight of 11,000. $K_{av}$ is an indicator of molecular weight and determined by the formula: (elution volume minus void volume)/(total volume minus void volume).

ND = not determined.

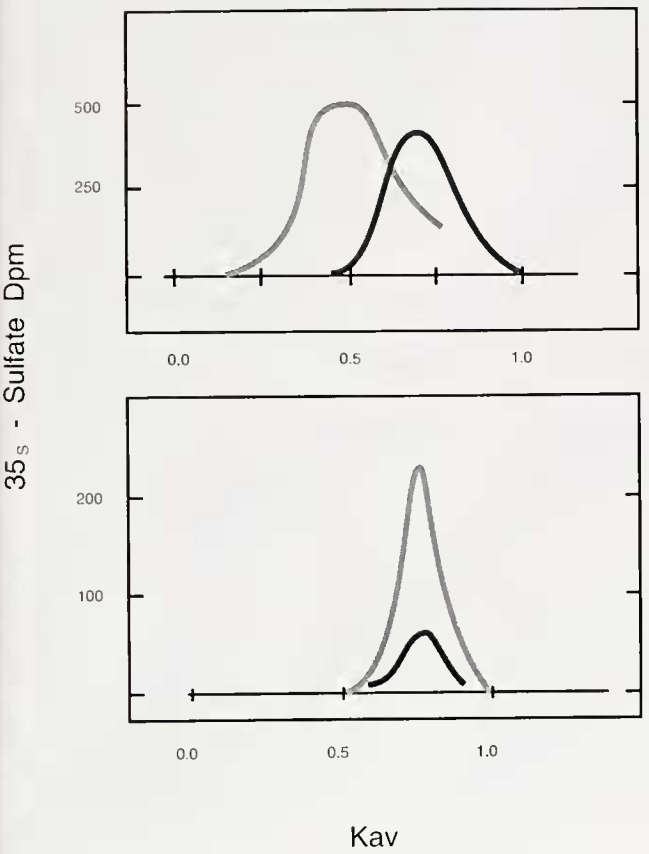



**Figure 2.** Sepharose CL-4B. Chromatography of material isolated from the peaks shown in Figure 1. Top: labeled material from gills in peak B before and after treatment with pronase. Solid line, untreated material; shaded line, pronase digested material. Bottom: labeled material from mantle in peaks A, B, C shown in Figure 1 after alkaline borohydride treatment. Material from peak A, shaded line; peak B, and peak C, solid line. Note that compared to Figure 1, material in peaks A and B shifted to a $K_{av}$ of 0.75–0.80, whereas material in peak C (with a $K_{av}$ of 0.75–0.80) showed no further shift.

trophoresis in 1.6 *M* formic acid at pH 1.6. Scintillation counting was performed on a Packard Tri Carb 1500 using Opti Fluor scintillation cocktails.

The presence of proteoglycans was determined by alkaline degradation with borotritide in 0.5 *N* NaOH for 16 h at 4°C or in 0.1 *N* NaOH for 16 h at room temperature. In this connection, the term 'free chains' (vs. proteoglycans) is used here as defining GAGs that show no change in molecular weight upon alkaline treatment; they may contain a small peptide, however.

*Organ culture*

Gills and mantles were obtained from the mussels after the adductor muscles were transected (to open the shell). Organs were placed in the medium in Falcon 3046 multiwell (35 mm) tissue culture plates (Becton Dickinson).

**Table II**

*Distribution of molluscan GAG (Fraction C) on ion-exchange chromatography and on cellulose acetate electrophoresis*

| | NaCl molarity | Gills | Mantle |
|---|---|---|---|
| *Ion exchange* | 1.0 *M* | 16% | 19% |
| | 1.25 *M* | 38% | 45% |
| | 1.5–2.0 *M* | 46% | 33% |
| *Electrophoresis* | Migrating as: | | |
| | Heparan sulfate | 55% | 79% |
| | Heparin | 45% | 21% |

[$^{35}$S] sulfate alone, or with up to 50 μCi/ml radiolabeled glucosamine, 5 μCi/ml. The tissues were slowly shaken in a water bath at 25°C for 6 or 24 h and then washed with cold medium and ground in a Potter-Elvehjem tissue grinder in the presence of 1% Triton X-100 in 4 *M* urea containing the protease inhibitors. The ground material was added to a DEAE-Sephacel column and washed extensively with the above solution in 0.2 *M* NaCl. The labeled material was then eluted with the 1% Triton, 4 *M*

**Table III**

*Composition of radiolabeled sulfate polymers in molluscan gills and mantle*

| | "Heparin"* | Percent chondroitin sulfate* | Other* |
|---|---|---|---|
| *Mantle* | | | |
| Total | | | |
| Expt I | 58 | 0 | 42 |
| Expt II | 96 | 1 | 3 |
| Fraction A | | | |
| Expt I | 0 | 0 | 100 |
| Expt II | 50 | 0 | 50 |
| Fraction B | | | |
| Expt I | 21 | 0 | 79 |
| Expt II | 80 | 0 | 20 |
| Fraction C | | | |
| Expt I | 75 | 0 | 25 |
| Expt II | 98 | 1 | 1 |
| *Gill* | | | |
| Total | | | |
| Expt I | 72 | 4 | 26 |
| Expt II | 84 | 1 | 15 |
| Fraction A | | | |
| Expt I | 46 | 8 | 46 |
| Expt II | 0 | 0 | 100 |
| Fraction B | | | |
| Expt I | 29 | 0 | 71 |
| Expt II | 17 | 0 | 83 |
| Fraction C | | | |
| Expt I | 81 | 0 | 19 |
| Expt II | 86 | 1 | 13 |

* Identified by heparinase, chondroitinase, and nitrous acid.

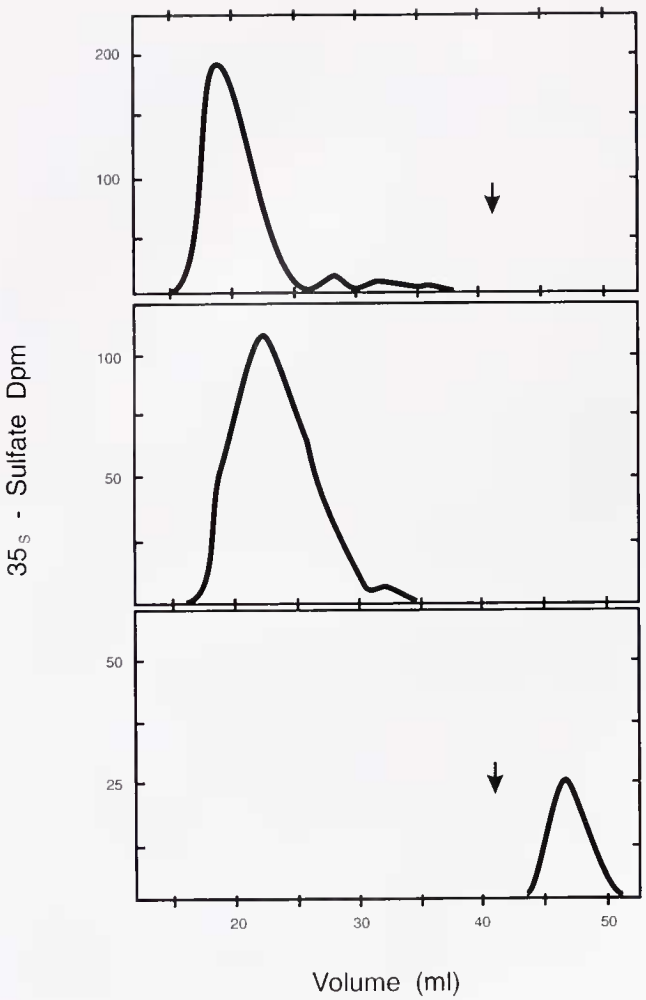



**Figure 3.** Characterization of GAGs of the mantle in peak C of Figure 1. Chromatography on Sephadex G-50 of the GAGs treated with heparitinase (top) purified heparinase (middle), and nitrous acid (bottom).

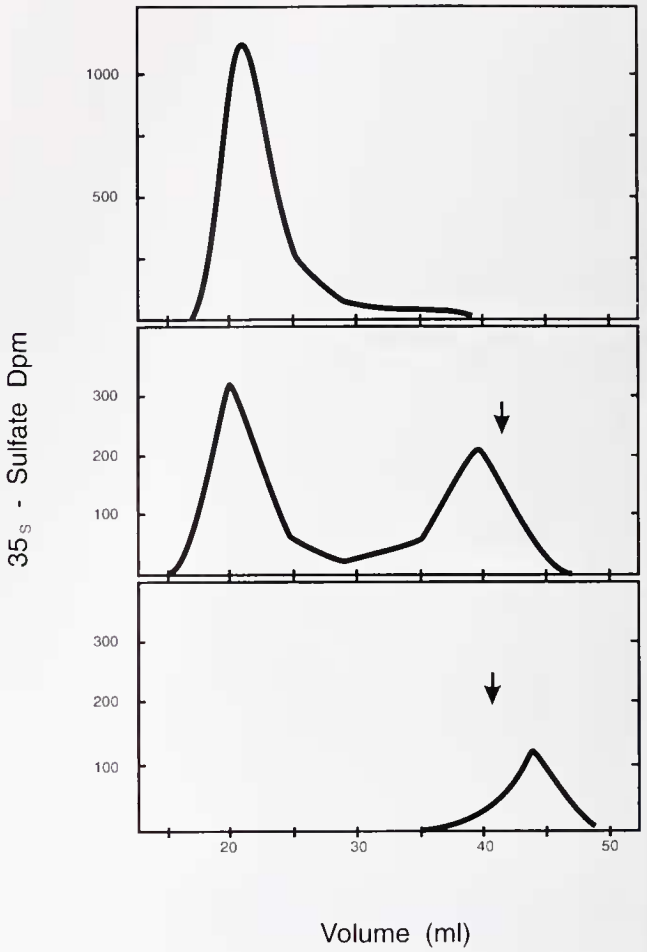



**Figure 4.** Characterization of GAGs of gills in peak C of Figure 1. Chromatography on Sephadex G-50 of the GAGs treated with heparitinase (top), purified heparinase (middle), and nitrous acid (bottom).

urea solution containing 1.0 *M* NaCl and characterized further, as will be described.

*Histology*

Parts of the incubated tissues were processed in a routine manner with an Autotechnicon processor, embedded in paraffin, and sectioned. Crude flavobacterial heparinase (Linker and Hovingh, 1972) was used for the identification of sulfate-labeled material. The enzyme, at 1 mg/ml in phosphate-buffered saline, was added to the deparafinized sections on the slides and incubated for 1 h at 25°C just prior to staining. The saline was used only in control incubations. The sections were examined by autoradiography using Kodak NTB2 emulsion and exposure for 3–5 days. They were then stained and counterstained, respectively with either Alcian blue and Congo red or with hematoxylin and eosin.

## Results

The labeled polymers isolated after organ culture were first examined for size, charge, and carbohydrate composition. Size distributions on Sepharose CL-4B are shown

**Table IV**

*Structural studies of molluscan "heparin" in Fraction C*

| | Percent degraded (radiolabeled sulfate) | | |
|---|---|---|---|
| | Heparitinase | Heparinase | Nitrous Acid* |
| *Gills* | | | |
| Expt I | 3** | 52 | 31 |
| Expt II | ND | 27 | 59 |
| *Mantle* | | | |
| Expt I | 22** | 13** | 51 |
| Expt II | ND | 28 | 70 |

* Material resistant to heparinase degraded further by nitrous acid.
** No disaccharides or tetrasaccharides.
ND = not determined.

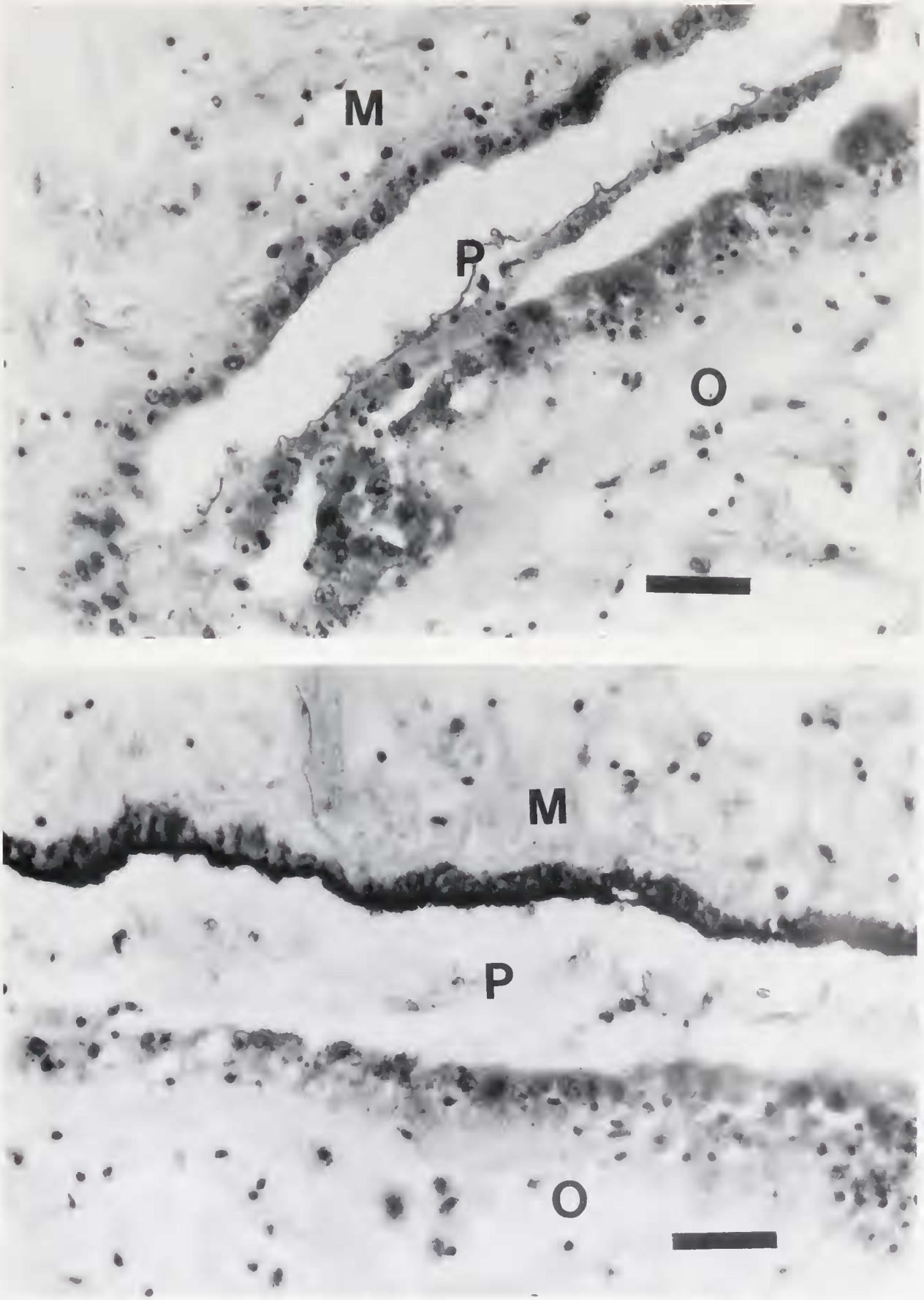
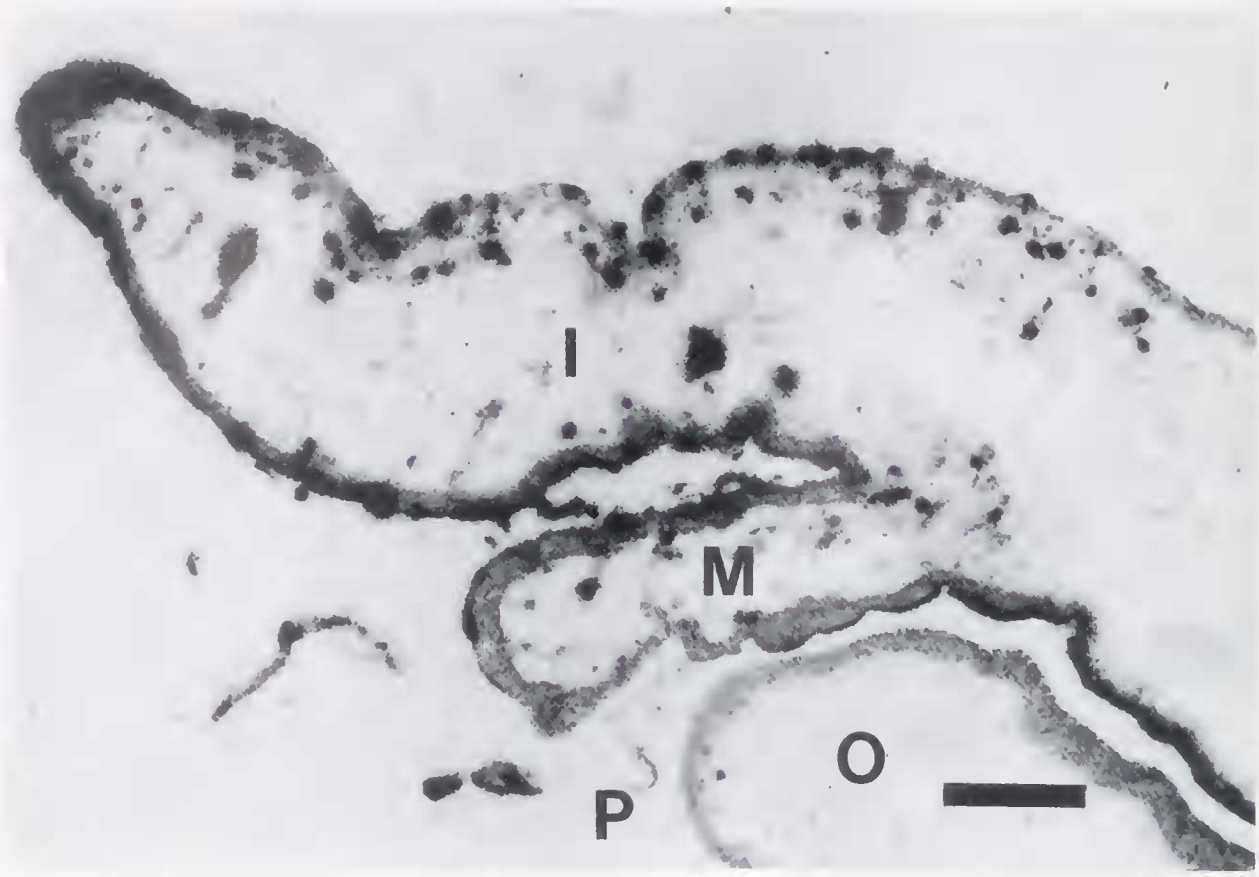



**Figure 5.** Mantle section. A view of the middle fold (M) and outer fold (O) with the periostracum (P) between the two folds. Magnification × 25, stained with hematoxylin and eosin. Upper figure from unlabeled mantle and lower figure mantle incubated with labeled sulfate and developed for autoradiography. Bar = 50 $\mu$m.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M
O
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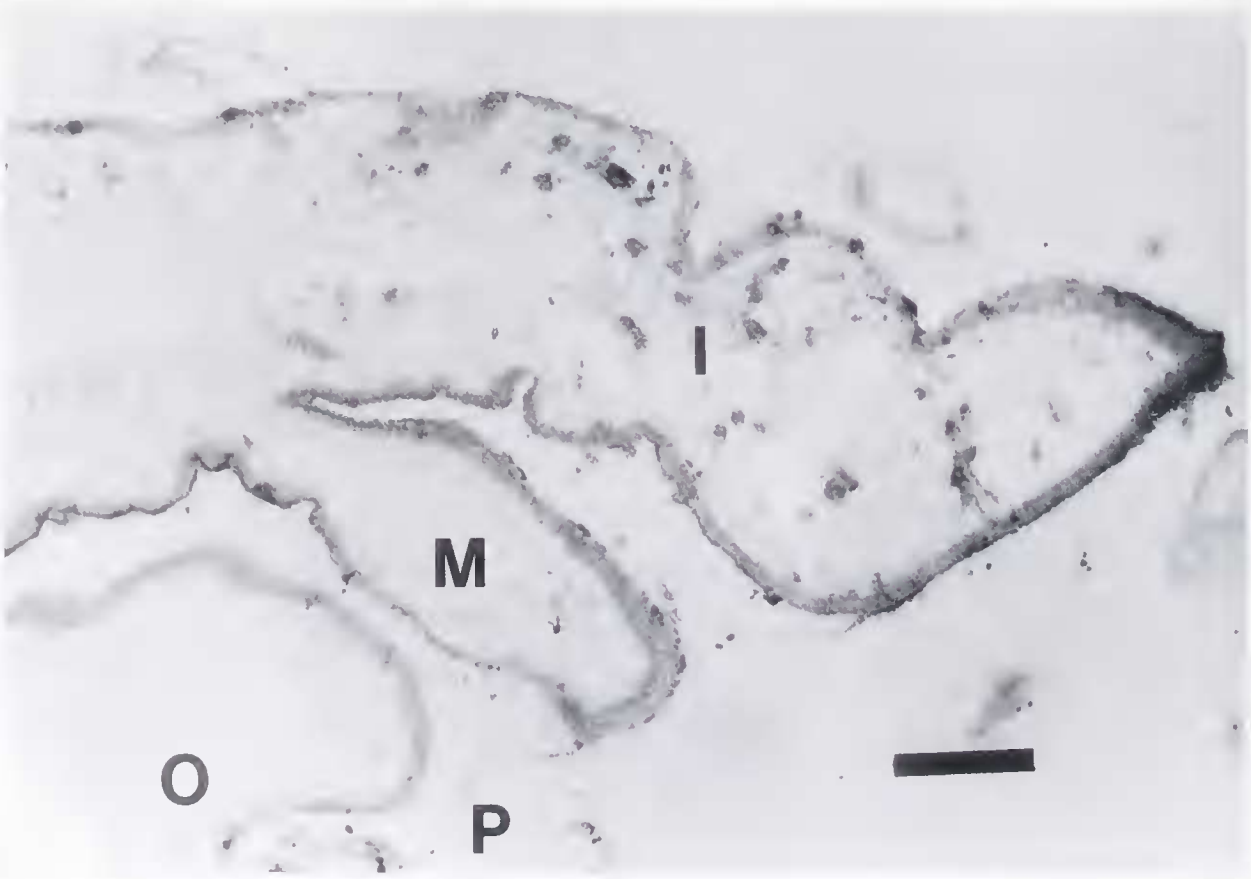
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in Figure 1 and Table I for material from mantle and gills. Size is measured as $K_{av}$, an indicator of molecular weight, calculated as (elution volume − void volume) ÷ (total volume − void volume). Two minor and one major peak can be seen for the sulfate-labeled polymers. After pronase or alkaline borohydride treatment, most of the minor fractions A and B shifted to a $K_{av}$ of 0.75–0.80 (similar to fraction C), indicating that a part may consist of proteoglycans (Fig. 2). Material in peak C showed no change in size after treatment, indicating that it consists of free GAG chains; this is further supported by its elution close to commercial heparin ($K_{av}$ = 0.77). Distribution of sulfate-labeled compounds into peaks A, B, and C is shown in Table I. The variation in distribution in the three experiments is most likely due to the physiological state of the mussels, which could vary depending on when the animals were collected and whether they were used immediately or kept in the laboratory before use. On ion exchange chromatography (AGI × 2), the fraction of peaks A and B that was resistant to alkaline borohydride and pronase eluted with 0.5 *M* NaCl. This fraction contained labeled sulfate and glucosamine, but was not GAG in nature because it was not degraded by heparinase, chondroitinase, or nitrous acid.

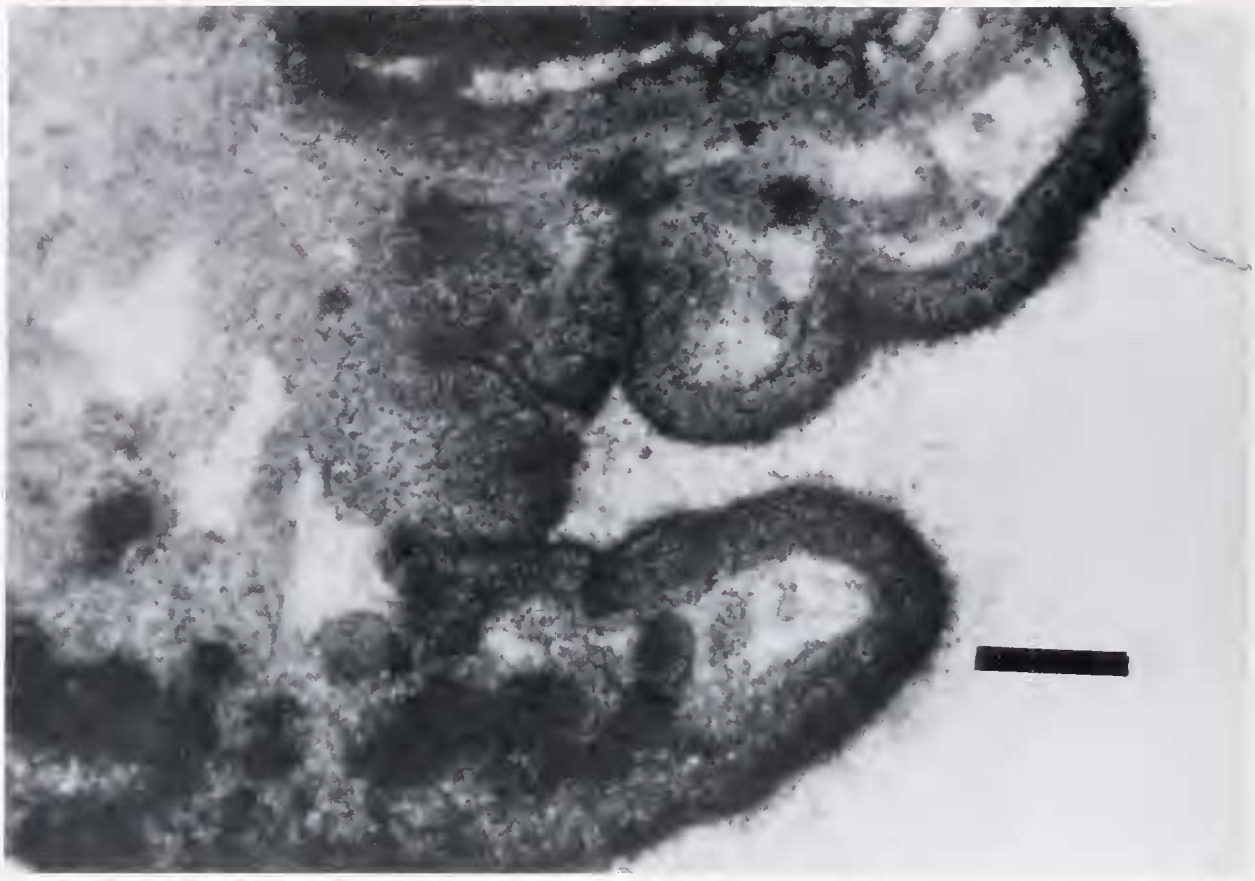
The polymers in peak C, the major fraction, were eluted from the ion exchange resin as shown in Table II. Note that a larger percentage of the GAGs from gill eluted at the highest salt concentration, indicating a higher charge content. Cellulose acetate electrophoresis confirmed the finding from ion exchange chromatography that the GAG from mantle contained a considerably higher percentage of material with a lower sulfate content, as does heparan sulfate (Table II).

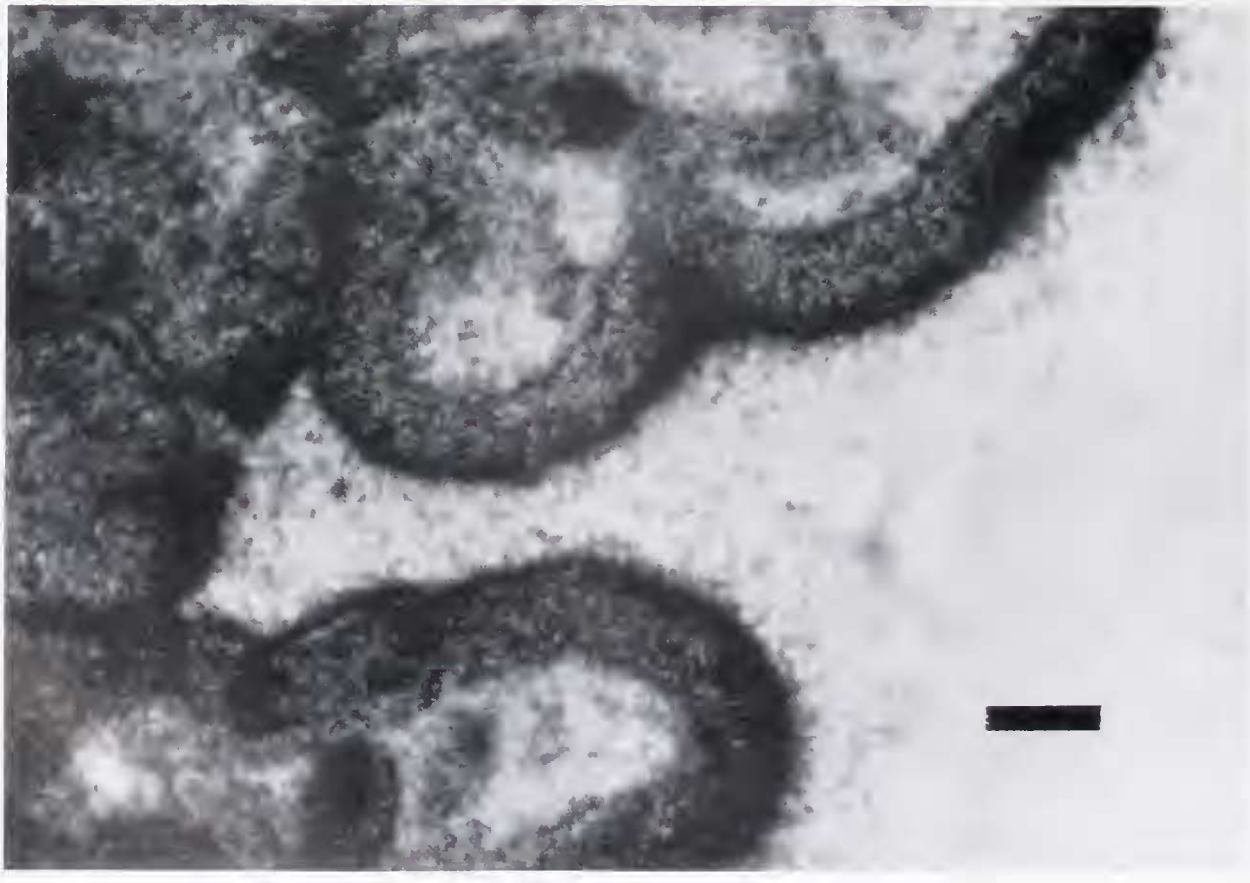
The GAG composition of sulfate- and glucosamine-labeled material in peaks A, B and C was further determined by degradation with nitrous acid, heparinase, chondroitinase ABC, and electrophoresis. As seen in Table III, the major components migrated, like heparin and heparan sulfate, with little chondroitin sulfate; but fairly substantial, though variable, amounts of non-GAG polymers in the minor peaks A and B. The variations between experiments could result from differences in the physiological states of the mussels.

Structural characteristics of material eluting in the Sepharose peak C, which migrated like heparin and heparan sulfate on electrophoresis, were examined by degradation with nitrous acid, heparinase, and heparitinase. As shown in Figure 3 for mantle- and Figure 4 for gill-derived GAGs, nitrous acid extensively degraded the polymers present, a characteristic of heparin, which contains mainly N-sulfated glucosamine, and to a lesser extent of heparan sulfate, which usually contains about 50% N-acetyl glucosamine and is therefore degraded less extensively. The mantle GAGs were degraded to a small extent by heparitinase and heparinase; although the gill polymers were barely degradable by heparitinase, 50% was extensively degraded to low molecular weight oligosaccharides by heparinase (Figs. 3 and 4). Quantitation of the data is shown in Table IV.

The nitrous acid reaction and the specificity of the enzymes used allows some structural information to be deduced. The heparitinase does not act on standard heparin or heparin-like segments of heparan sulfate. It degrades mono- or non-sulfated sequences of heparan sulfate. The heparinase requires N-sulfate and uronic acid sulfate repeating units for activity and will therefore act well on standard heparins and on heparin-like segments of heparan sulfate, if present (Linker and Hovingh, 1972; Hovingh and Linker, 1974). That is, the mantle polysaccharides, which are degraded to small oligosaccharides by nitrous acid, must have mainly N-sulfated glucosamine and additional O-sulfate to be fairly resistant to heparitinase, but may not contain sufficient O-sulfated iduronic acid to be susceptible to heparinase. Extensive degradation by nitrous acid indicates the absence of N-acetyl. The mantle polysaccharides therefore resemble the "heparan sulfate" isolated from lobsters (Hovingh and Linker, 1982). Approximately half of the GAGs from gills are well degraded by heparinase and are resistant to heparitinase. This, combined with the finding of degradation by nitrous acid, indicates that at least half of this gill product closely resembles vertebrate heparins, but consists mostly of disulfated repeating units. Thomas (1954) reported the isolation of two types of metachromatic GAGs from mucous and tissues of the surf clam *Spisula*, with only one type having significant anticoagulant activity. The presence of sulfated non-GAG polymers (see fractions A and B) has been reported for other species (Mourao and Bastos, 1987; Mourao and Perlin, 1987). The products obtained from nitrous acid treatment consisted of monosulfated disaccharides and tetrasaccharides derived from di-sulfated disaccharides and tetrasaccharides in the original polymer.

**Figure 6.** Section of the outer edge of the mantle. Interior fold (I), middle fold (M), outer fold, adjacent to shell (O), periostracum (P). Magnification × 10, stained with Alcian blue and Congo red. Mantle was incubated with labeled sulfate. Lower figure: section was treated with crude heparinase before autoradiography. Upper figure: control, not treated with enzyme. Bar = 400 μm.





*Histology*

The precise location of heparin in the tissues of the mussel is prerequisite to an understanding of the role of the polymer. We therefore carried out the histological studies described below. Our main approach was to examine sections of tissues that had been incubated with $^{35}SO_4$-labeled precursors and to locate the labeled polymers by autoradiography. Tissues incubated without the label were used as controls when needed.

Figure 5 shows a section of the mantle. The middle fold, outer fold, and periostracum can be seen. The labeled section shows the presence of sulfated GAGs on the periphery of the epithelial cells in the middle fold but not in the outer fold. Figure 6 represents the edge of the mantle showing the interior fold, middle fold, outer fold (adjacent to shell), and periostracum. The $^{35}SO_4$-labeled material was largely associated with the epithelium of the interior and middle fold, but not the outer fold. The labeled polymers were almost completely removed by crude heparinase, indicating that they consist of GAGs. Furthermore, because the isolation data showed that chondroitin sulfates are present only to a minor extent, the polymers must be related to heparin. The crude heparinase used combines the activities of heparitinase and purified heparinase and also contains another enzyme (heparitinase II), which acts on segments of heparan sulfate or heparin with an intermediate sulfate content, *i.e.*, too high for heparitinase I action and too low for the heparinase (Nader *et al.*, 1990). Figure 7, at a higher magnification, shows the location of the labeled GAGs more clearly. Note the preponderance of sulfate label in the outer pericellular portion of the epithelial cells, with a trace of label at the base of the epithelia. Also note (bottom figure) the dual layer of labeled GAGs at the outer pericellular location of the epithelial cells.

Figure 8 shows the interior fold of the mantle, including mucous cells and epithelium. An unlabeled section is included as control. The label is associated largely with the mucous cells and the epithelial cells. Gill sections are shown in Figure 9. Most of the labeled GAGs are associated with the filaments and not with the water tubes. The labeled polymers were largely removed by crude heparinase, indicating that they are heparin-like GAGs. Figure 10 shows the gill filaments at a higher magnification. Note the $^{35}SO_4$ label on the exterior pericellular surfaces of the filaments and some double membranes, which are labeled (see arrows). Some label occurs at the base of the cells.

## Discussion

The presence of GAGs in representatives of most major taxa from bacteria to mammals and the preservation of their main structural features during evolution indicates a fundamental role for these complex anionic polysaccharides in most biological systems. Some members of the six or so distinct GAGs have been shown to be present in Eubacteria (Sugahara *et al.*, 1979; Vann *et al.*, 1981; Rodriguez *et al.*, 1988); in the invertebrate phyla Porifera, Coelanterata, Annelida, Arthropoda, Mollusca, Echinodermata, and in the invertebrate members of the Chordata (Cassaro and Dietrich, 1977; Nader *et al.*, 1983; Nader *et al.*, 1984); and in all Vertebrata examined. Though the survey has not been exhaustive, the data are consistent with an extensive presence.

Among the GAGs, heparin and heparan sulfate form a subgroup of closely related but distinct polymers. These polymers are unique in that they contain N-sulfated glucosamine and have a highly variable structure. Heparin the most highly sulfated GAG, has an unusually sporadic phyletic distribution. Though it is present in some invertebrates (De Meio *et al.*, 1967; Rahemtulla and Løvtrup, 1975), heparin appears only occasionally in vertebrates (Gomes and Dietrich, 1982; Hovingh *et al.*, 1986), and though synthesized by most mammals, it is absent in rabbits and present in only small amounts in man. Heparan sulfate, on the other hand, has one of the widest distributions known. Heparin was isolated from marine bivalves in amounts adequate for detailed structural investigations. Except for some minor differences, it is very close to mammalian heparins in structure and has a high anticoagulant activity (Pejler *et al.*, 1987).

In keeping with the wide distribution of these compounds, a large variety of biological functions have been assigned to GAGs or, more precisely, to their proteoglycans. Aside from their role in vertebrate connective tissue, they appear to interact with growth factors, tissue matrix components, and cell receptors, and may play a role in cell migration, morphology, and angiogenesis (Kjellen and Lindahl, 1991).

As for heparin, it is certainly an excellent anticoagulant when used as a pharmacological agent, but its location in the animals studied (*i.e.*, most likely in the mast cell only) seems to preclude this as its true biological activity. Other well-documented *in vitro* interactions—with growth factors, cell receptors, and matrix components such as laminin or fibronectin—may not occur *in vivo*, because hep-

**Figure 7.** Section of the mantle at higher magnification. Magnification × 25; bar = 50 μm (upper figure) and × 40; bar = 20 μm (lower figure). Incubated with labeled sulfate. Developed for autoradiography, and stained with hematoxylin and eosin. Note the preponderance of sulfate label in the outer pericellular location of the epithelial cells and a trace of radioactivity at the base of the cells. In the bottom figure, note the dual layer of label on the outer pericellular location.

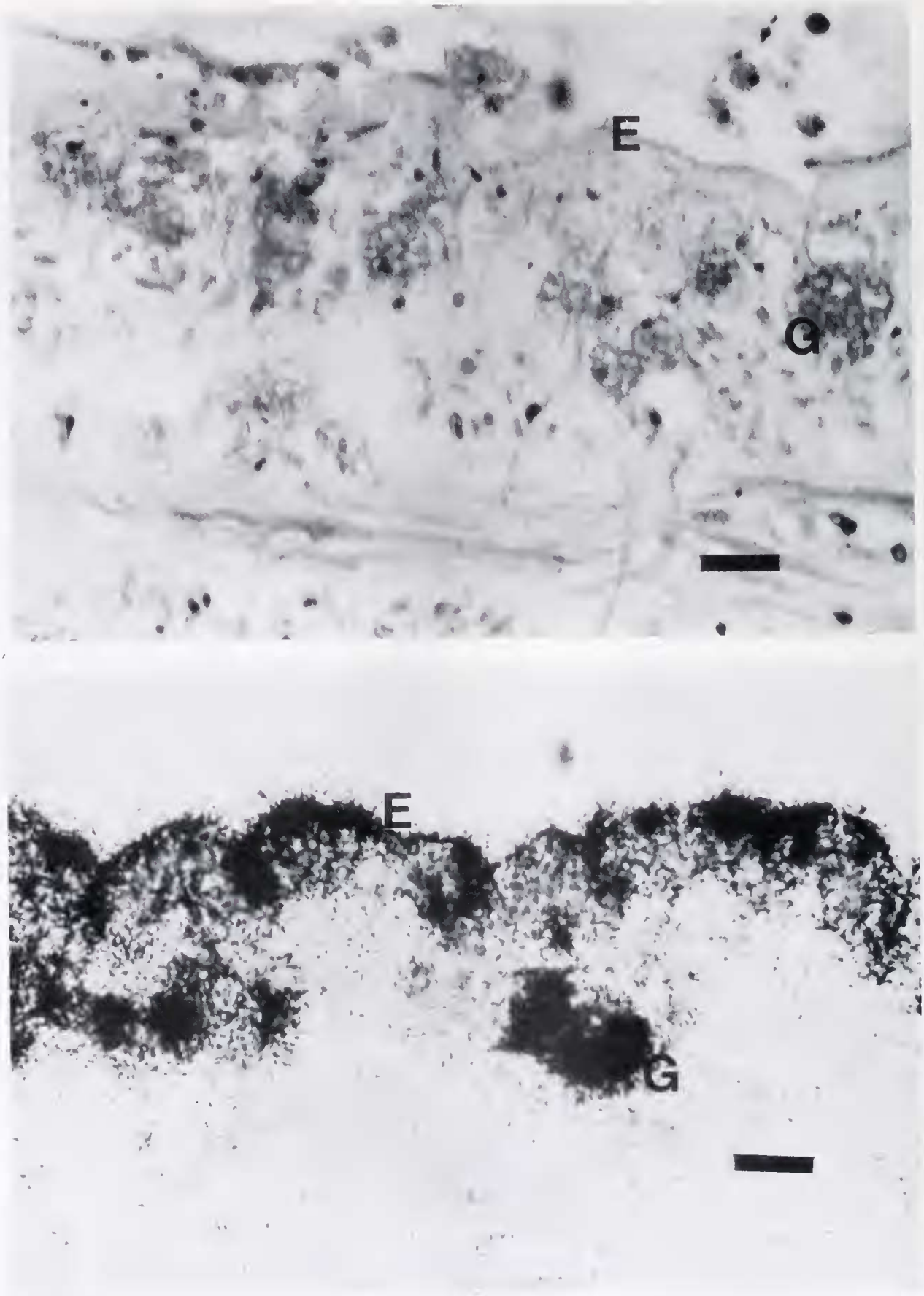



**Figure 8.** Section of the interior fold showing mucous cells (G) and epithelium (E). Magnification × 40; Bar = 20 μm. Stained with hematoxylin and eosin after autoradiography. Upper figure: unlabeled mantle. Lower figure: incubated with radiolabeled sulfate.

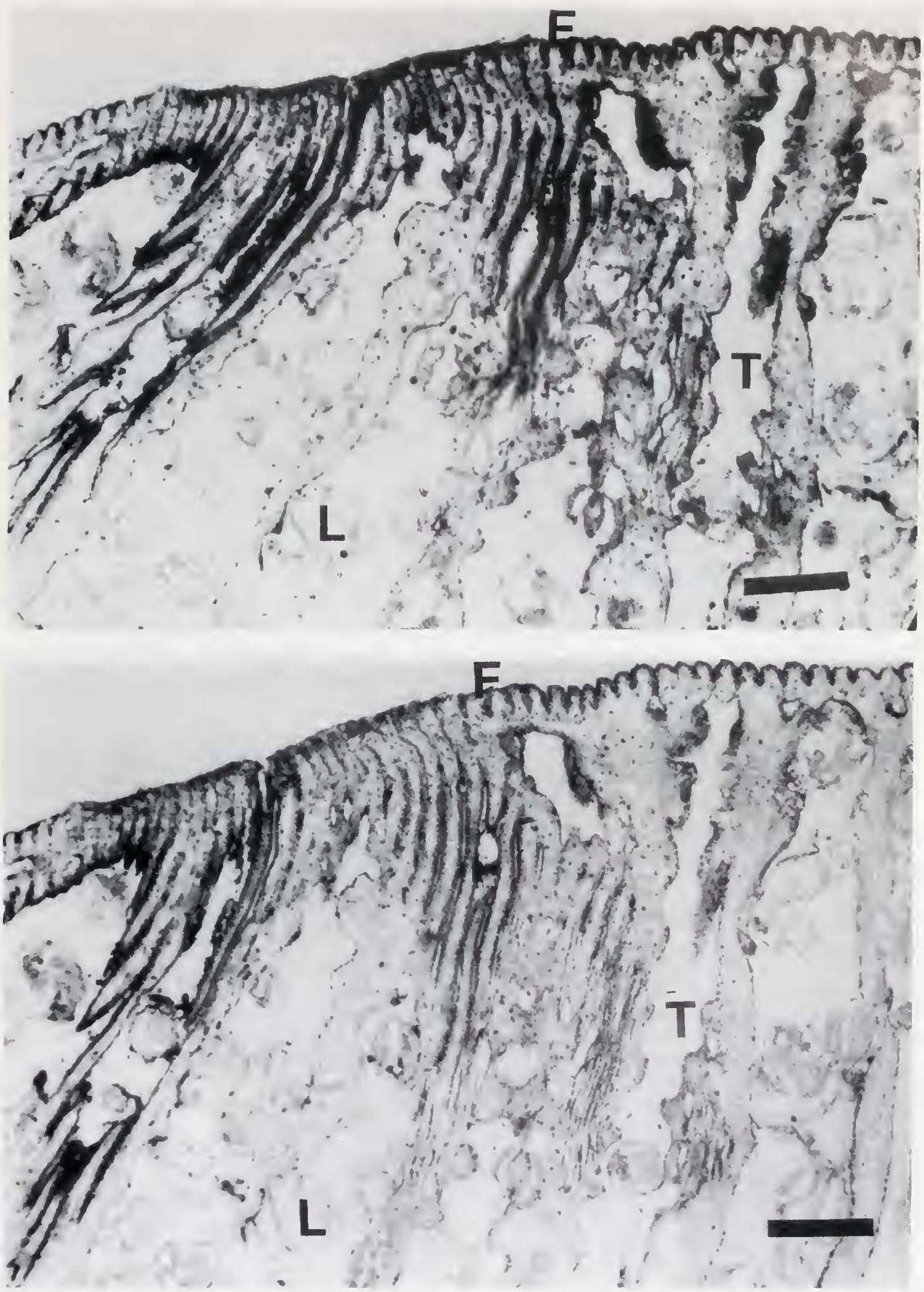



**Figure 9.** Section of the gills. Magnification × 4; Bar = 400 μm. Note filaments (F); water tubes (T), and larva (L). Stained with Alcian blue and Congo red after autoradiography. Gills were incubated with labeled sulfate. Lower figure: section was treated with crude heparinase. Upper figure: control not treated with enzyme.

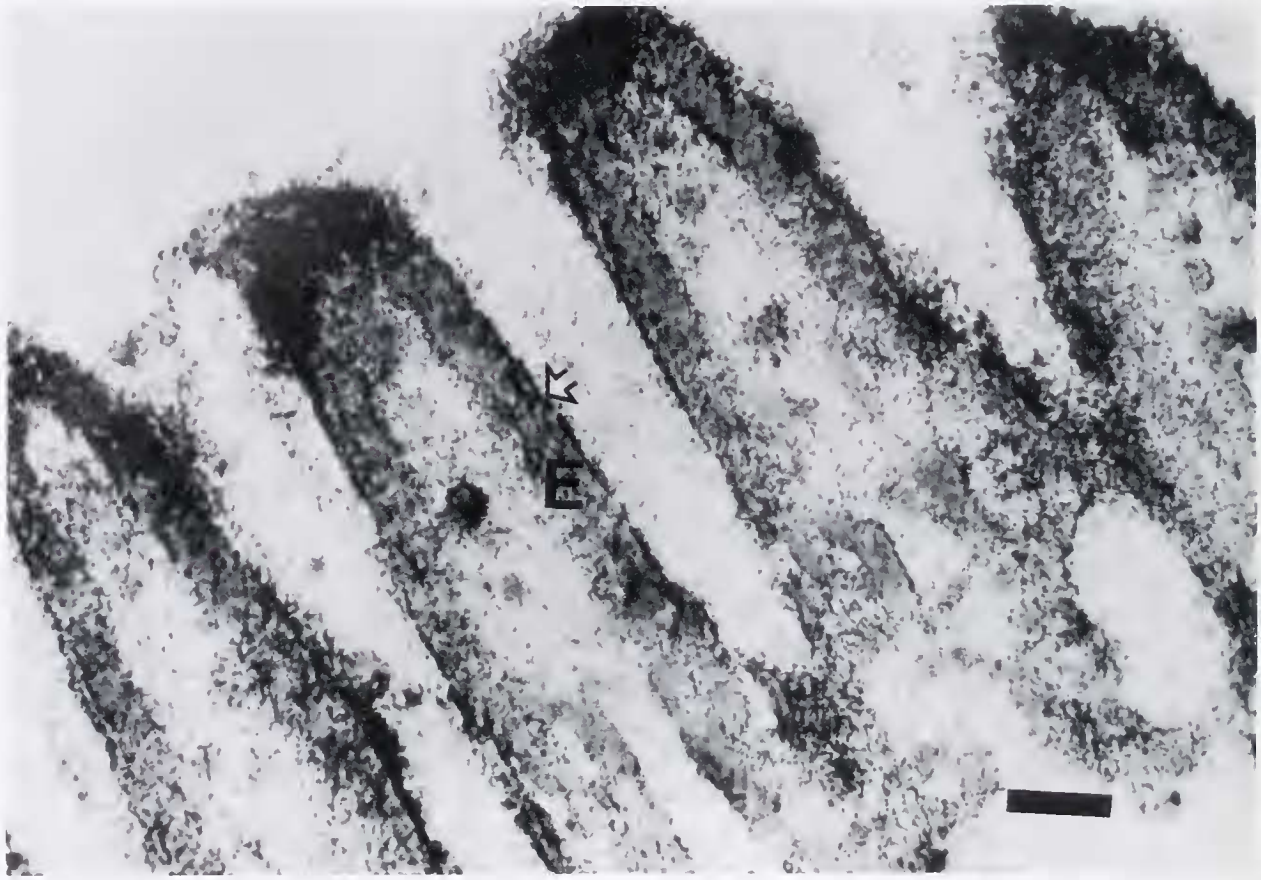



**Figure 10.** Section of gills filaments. Magnification × 40; Bar = 20 μm. Gills were incubated with labeled sulfate. Stained with hematoxylin and eosin after autoradiography. epithelial layer (E). Note the sulfate label on the exterior pericellular surfaces of the filaments (arrow) and the occasional double membranes which are labeled. Some label occurs at the base of the cells.

arin is not present in locations where such interactions would be plausible. Whatever the biological role of heparin may be, it is not clear, however, what other substance fulfills the same role in species that lack this apparently quite critical member of the GAG family. There is no adequate evidence that heparan sulfate substitutes for heparin in such cases. If structural consistency implies functional consistency, a study of the function of heparin or heparin-like compounds in invertebrates such as clams may give a clue to the role of these GAGs in vertebrates.

In this study we have shown the synthesis of heparin and a heparin- or heparan-sulfate-like polysaccharide in a freshwater mussel. Though the synthetic pathway may involve a proteoglycan precursor, the major final product, as in mammals, is a free GAG chain (see Table I). The data obtained by the use of specific degradative enzymes and nitrous acid treatment show that gills synthesize approximately 50% each of one polymer that shows the characteristics of a somewhat poorly sulfated heparin (see Tables II, III, and IV) and a second polymer intermediate in structure to heparan sulfate and heparin. Little heparin appears to be synthesized in the mantle, where the major material labeled is the intermediate polymer, which seems to be closely related to a heparan-sulfate-like GAG isolated from lobsters (Hovingh and Linker, 1982). Large-scale preparation could clarify this similarity further, but *Anodonta californiensis* occurs only in small and isolated populations. Other species of *Anodonta,* or other uniodid clams, are more plentiful and thus more suitable for scaling up the preparation, provided that the distribution of GAGs is similar to that in *A. californiensis.*

The histology shows the presence of heparin or heparan sulfate in the mantle and gills, *i.e.,* in the exterior pericellar and "basement membrane" locations of the epithelial cells and on the exterior surface of gill filaments; that is, in areas exposed to the aquatic medium. GAGs in general have been implicated in $Ca^{2+}$ metabolism and calcification (Sauren *et al.,* 1992) and heparin has been shown to complex with $Ca^{2+}$ (Grant *et al.,* 1992) and to bind strongly to L-type $Ca^{2+}$ channels of vertebrate skeletal muscles (Knaus *et al.,* 1990). We propose a model in which heparin in mussels sequesters $Ca^{2+}$ from the aquatic medium and

transports it across the epithelial layer in the mantle and gills (and perhaps all organs with a surface bordering the environmental water). Heparin may not actually be involved in the transport itself, but only in the sequestering of $Ca^{2+}$ on both sides of the epithelial layer. After $Ca^{2+}$ is transported across the epithelial layer, $Ca^{2+}$ (without heparin) is accumulated and stored in spheroids for use in glochidia development in the gills (Silverman *et al.*, 1983, 1985, 1987) and in the calcification of the shell (Wilbur and Saleuddin, 1983). This model suggests that, as filter feeders, all bivalves (marine and freshwater), would use heparin-like GAG to efficiently sequester $Ca^{2+}$, whereas gastropods would obtain $Ca^{2+}$ in their food, so that heparin (if present) may have a different role than in bivalves. The model also implies that heparin will be found on the exterior surfaces of these organisms.

The model presented here could be tested by labeling mussels *in vivo* with $^{35}S$, treating them and the controls with $^{45}Ca$ and the crude Flavobacter enzymes, and following the disappearance of heparin from the epithelial surface and the incorporation of $^{45}Ca$ from the water.

Another role for heparin may be inactivation of toxic amines present in the aquatic environment: this is analogous to a function proposed for mast-cell heparin in vertebrates. As in clams, tunicates, and sea cucumbers (Cifonelli and Mathews, 1972, Mourao and Bastos, 1987; Mourao and Perlin, 1987), sulfate-labeled material in addition to GAGs was also isolated from the mantles and gills of the *Anodonta* (see Table 3). The data obtained are similar to those in earlier reports showing that heparin and other metachromatic polymers occur in the mantle, gills, and some other organs of mussels (Thomas, 1954; Nader and Dietrich, 1989). The functions and detailed structures of these sulfated polymers are unknown, although their molecular weight is high and they may be bound to threonine (Cifonelli and Mathews, 1972).

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## Literature Cited

**Cassaro, C. M. F., and C. P. Dietrich. 1977.** Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem.* **252:** 2254–2261.

**Cifonelli, J. A., and M. B. Mathews. 1972.** Structural studies on spisulan: a mucopolysaccharide from clams. *Connective Tissue Res.* **1:** 231–241.

**De Meio, R. H., Y.-C. Lin, and S. Narasimhulu. 1967.** Some aspects of the biosynthesis of mactin. *Comp. Biochem. Physiol.* **20:** 581–591.

**Gomes, P. B., and C. P. Dietrich. 1982.** Distribution of heparin and other sulfated glycosaminoglycans in vertebrates. *Comp. Biochem. Physiol.* **73B:** 857–863.

**Grant, D., W. F. Long, C. F. Moffat, and F. B. Williamson. 1992.** A study of $Ca^{2+}$-heparin complex-formation by polarimetry. *Biochem J.* **282:** 601–604.

**Hovingh, P., and A. Linker. 1974.** The disaccharide repeating-units of heparan sulfate. *Carb. Res.* **37:** 181–192.

**Hovingh, P., and A. Linker. 1982.** An unusual heparan sulfate isolated from lobsters (*Homarus americanus*). *J. Biol. Chem.* **257:** 9840–9844.

**Hovingh, P., M. Piepkorn, and A. Linker. 1986.** Biological implications of the structural, antithrombin affinity and anticoagulant activity relationships among vertebrate heparins and heparan sulphates. *Biochem. J* **237:** 573–581.

**Kjellen, L., and U. Lindahl. 1991.** Proteoglycans: structures and interactions. *Ann. Rev. Biochem.* **60:** 443–475.

**Knaus, H.-G., F. Scheffauer, C. Romanin, H.-G. Schindler, and H. Glossmann. 1990.** Heparin binds with high affinity to voltage-dependent L-type $Ca^{2+}$ channels: Evidence for an agonistic action. *J. Biol. Chem.* **265:** 11156–11166.

**Linker, A., and P. Hovingh. 1972.** Heparinase and heparitinase from Flavobacteria. Pp. 902–911 in *Methods in Enzymology*. U. Ginsburg, ed. Academic Press, New York.

**Mourao, P. A. S., and I. G. Bastos. 1987.** Highly acidic glycans from sea cucumbers: Isolation and fractionation of fucose-rich sulfated polysaccharides from the body wall of *Ludwigothurea grisea*. *Eur. J. Biochem.* **166:** 639–645.

**Mourao, P. A. S., and A. S. Perlin. 1987.** Structural features of sulfated glycans from the tunic of *Styela plicata* (Chordata—Tunicata): A unique occurrence of L-galactose in sulfated polysaccharides. *Eur. J. Biochem.* **166:** 431–436.

**Nader, H. B., and C. P. Dietrich. 1989.** Pp. 81–96 in *Heparin, Chemical and Biological Properties*. D. A. Lane and U. Lindahl eds. CRC Press, Florida.

**Nader, H. B., T. M. P. C. Ferreira, J. F. Paiva, M. G. L. Madeiros, S. M. B. Jeronimo, V. M. P. Paiva, and C. P. Dietrich. 1984.** Isolation and structural studies of heparan sulfates and chondroitin sulfates from three species of molluscs. *J. Biol. Chem.* **259:** 1431–1435.

**Nader, H. B., M. G. L. Madeiros, J. F. Paiva, V. M. P. Paiva, S. M. B. Jeronimo, T. M. P. C. Ferreira, and C. P. Dietrich. 1983.** A correlation between the sulfated glycosaminoglycan concentration and degree of salinity of the "habitat" in fifteen species of the classes Crustacea, Pelecypoda and Gastropoda. *Comp. Biochem. Physiol.* **76:** 433–436.

**Nader, H. B., M. A. Porcionatto, I. L. S. Tersariol, M. A. S. Pinhal, F. W. Oliveira, C. T. Moraes, and C. P. Dietrich. 1990.** Purification and substrate specificity of heparitinase I and heparitinase II from Flavobacterium heparinum: Analyses of the heparin and heparan sulfate degradation products by $^{13}C$ NMR spectroscopy. *J. Biol. Chem.* **265:** 16807–16813.

**Pejler, G., A. Danielsson, I. Bjork, U. Lindahl, H. B. Nader, and C. P. Dietrich. 1987.** Structure and antithrombin-binding properties of heparin isolated from the clams *Anomalocardia brasiliana* and *Tivela mactroides*. *J. Biol. Chem.* **262:** 11413–11421.

**Rahemtulla, F., and S. Lovtrup. 1974.** The comparative biochemistry of invertebrate mucopolysaccharides—II. Nematoda: Annelida. *Comp. Biochem. Physiol.* **49B:** 639–646.

**Rahemtulla, F., and S. Lovtrup. 1975.** The comparative biochemistry of invertebrate mucopolysaccharides—IV. Bivalvia. Phylogenetic implications. *Comp. Biochem. Physiol.* **50B:** 631–635.

**Rodriguez, M.-L., B. Jann, and K. Jann. 1988.** Structure and serological characteristics of the capsular K4 antigen of *Escherichia coli* 05:K4:

H4, a fructose-containing polysaccharide with a chondroitin backbone. *Eur. J. Biochem.* **177**: 117–124.

**Sauren, Y. M. H. F., R. H. P. Mieremet, C. G. Groot, and J. P. Scherft. 1992.** An electron microscopic study on the presence of proteoglycans in the mineralized matrix of rat and human compact lamellar bone. *Anatom. Record* **232**: 36–44.

**Shively, J. E., and H. E. Conrad. 1976.** Formation of anhydro-sugars in the chemical depolymerization of heparin. *Biochemistry* **15**: 3932–3942.

**Silverman, H., W. T. Kays, and T. H. Dietz. 1987.** Maternal calcium contribution to glochidial shells in freshwater mussels (Eulamellibranchia: Unionidae). *J. Exper. Zoology* **242**: 137–146.

**Silverman, H., W. L. Steffens, and T. H. Dietz. 1983.** Calcium concretions in the gills of a freshwater mussel serve as a calcium reservoir during periods of hypoxia. *J. Exper. Zoology* **227**: 177–189.

**Silverman, H., W. L. Steffens, and T. H. Dietz. 1985.** Calcium from extracellular concretions in the gills of freshwater unionid mussels is mobilized during reproduction. *J. Exper. Zoology* **236**: 137–147.

**Sugahara, K., N. B. Schwartz, and A. Dorfman. 1979.** Biosynthesis of hyaluronic acid by *Streptococcus*. *J. Biol. Chem.* **254**: 6252–6261.

**Thomas, L. J., Jr. 1954.** The localization of heparin-like blood anticoagulant substances in the tissues of *Spisula soldissima*. *Biol. Bull.* **106**: 129–138.

**Vann, W. F., M. A. Schmidt, B. Jann, and Jann K. 1981.** The structure of the capsular polysaccharide (K5 antigen) of urinary tract-infective *Escherichia coli* 010:K5:H4. A polymer similar to desulfo-heparin. *Eur. J Biochem.* **116**: 359–364.

**Wilbur, K. M., and A. S. M. Saleuddin. 1983.** Shell formation. Pp. 235–287 in *The Mollusca*, Vol. 4, Physiology, Part I. Academic Press, New York.